

Triton X-100 Induces Apoptosis in Human Hepatoma Cell Lines

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The detergent Triton X-100 was used to establish a model for apoptosis in hepatoma cell lines. The electrophoresis of DNA extracted from 0.01% Triton X-100 treated hepatoma cell lines showed DNA ladder formation, a hallmark of apoptosis. The DNA fragmentation appeared within less than 60 min of the Triton X-100 treatment. Chromatin condensation and apoptotic bodies were observed by hematoxylin and eosin (H & E) stain, and fragmented nucleosome was detected by terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labelling (TUNEL) test. Apoptosis was semi-quantitated by measuring the lactate dehydrogenase (LDH) level for cytotoxicity. It was found that apoptosis had been induced in more than 90% of the cells treated with Triton X-100 for 150 min. These data show that Triton X-100 efficiently induces the apoptotic cell death in hepatoma cell lines.

Key Words: Triton X-100, apoptosis, hepatoma cells lines, DNA fragmentation, TUNEL test, LDH Introduction

Normal organisms require the regulation of cell viability, growth and differentiation. Apoptosis, which has also been called programmed cell death (PCD) in some biological fields, is a normal physiological phenomenon that can be observed in various tissues (Arends and Wyllie, 1991). Cell death may also be

subjected to mutagenic chemicals or radiation. When epithelial cells are exposed to carcinogens in the diet, cells die by physiological means (Duncan and Heddle, 1984). When the skin is exposed to mutagenic UV radiation, physiological cell death is used to remove epidermal cells, rather than letting them passively slough away (Young, 1987).

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Cell death by apoptosis is characterized by physiological and morphological changes. The characteristic morphological features of apoptosis include condensation of chromatin around the nuclear perimeter, cell shrinkage, cell membrane blebbing and release of apoptotic bodies from the cell (Fesus *et al.* 1989). Apoptotic DNA degradation produces DNA fragments in multiples of 185-200 bp length, which can be visualized as a distinct ladder pattern in DNA electrophoresis (Gaido and Cidrowski, 1991).

It has been proposed that apoptosis provides a protective mechanism whereby DNA-damaged or potentially neoplastic cells are selectively eliminated

(Nagata and Suda, 1995). Cytotoxic T lymphocytes and lymphokine-induced apoptosis of infected hepatocytes during the course of chronic viral hepatitis are thought to be important for both disease termination and prevention of hepatocellular transformation (Natoli *et al.* 1995). In hepatoma cells, apoptotic cell death may be activated by various agents through different pathways. *In vivo*, it was reported that chronic dietary restriction induced an increase in the spontaneous rate of apoptotic cell death in hepatocytes of B6C3F1 mice, and this phenomenon was associated with a significant development of spontaneous hepatoma (Muskhelishvili *et al.* 1995). Serum deprivation (Pandey *et al.* 1994), anticancer drugs (Evans and Dive, 1993; Kaneko and Tsukamoto, 1995), retinoids (Nakamura *et al.* 1995), transforming growth factor (TGF)- β (Fukuda *et al.* 1993; Bayly *et al.* 1994; Chuang *et al.* 1994), and Ca^{2+} -ATPase inhibitor (Tsukamoto and Kaneko, 1993; Kaneko and Tsukamoto, 1994) induced apoptosis *in vitro* in hepatoma cell lines.

To evaluate whether Triton X-100, a non-ionic detergent which is used to lyse cells, induces apoptosis in hepatoma cell lines, we treated several hepatoma cell lines with 0.01% Triton X-100. By analyzing DNA fragmentation and morphological features of the cells, we provide evidence that Triton X-100 can induce apoptosis in hepatoma cells.

MATERIALS AND METHODS

Human hepatoma cell lines and cell culture

Hepatitis B virus (HBV) non-productive human hepatoma cell lines, Hep 3B [ATCC HB 8064, HBs Ag(+)], Hep G2 [ATCC HB 8065, HBs Ag(-)], PLC/PRF/5 [ATCC CRL 8024, HBs Ag(+)], and SK-Hep-1 [ATCC HTB 52] were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). And Hep G2.2.15, a HBV productive hepatoma cell line (Puisieux *et al.* 1995), was also used. These cells were cultured with minimal essential medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin (Gibco BRL, Grand Island, NY, USA).

Treatment of cells with Triton X-100

A total of 2×10^6 cells were plated onto the 10 cm culture dish. After overnight incubation, the culture medium was replaced with prewarmed fresh complete medium. After 4 hr incubation, the culture medium was replaced with a fresh serum-free medium to prevent any possible interaction of Triton X-100 with serum proteins. A 2% stock solution of Triton X-100 (w/v, Boi-Rad Laboratories, Hercules, CA, USA) was added to the serum-free culture medium to make a final concentration of 0.01% (Borner *et al.* 1994), then the culture was terminated after various incubation intervals, 30, 60, 90, and 150 min.

Assay of DNA fragmentation

The cells treated with 0.01% Triton X-100 were collected and washed with 5 ml phosphate buffered saline (PBS) and resuspended with 200 μl PBS, then 1 ml of lysis buffer (10 mM Tris, pH 7.6, 10 mM EDTA, 50 mM NaCl, 0.2% SDS and 200 $\mu\text{g}/\text{ml}$ proteinase K) was added. After overnight incubation at 42°C with gentle agitation, cell lysate was extracted with phenol/chloroform. The DNA was precipitated with isopropanol and separated in 1.2% agarose gel containing 0.5 $\mu\text{g}/\text{ml}$ ethidium bromide. Gels were photographed under the UV light with Polaroid 57 type film (Sambrook *et al.* 1989).

Hematoxylin and eosin stain (H & E stain)

After treatment of the cells with 0.01% Triton X-100, they were collected and washed with PBS, and fixed with formalin. Then a paraffin-embedded cell block was made and H & E stain was performed.

Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labelling (TUNEL) test

The TUNEL test was done with the ApopTaq™ kit (Oncor Inc., Gaithersburg, MD, USA) according to the manufacturer's instructions. In brief, a 50 μl cell suspension of 5×10^7 cells/ml, fixed with 4% paraformaldehyde for 10 min at room temperature (RT), was air dried on a microscopic slide, then

$$\% \text{ cytotoxicity} = \frac{\text{sample OD} - \text{spontaneous OD}}{\text{total OD} - \text{spontaneous OD}} \times 100$$

Spontaneous OD was obtained from the supernatant of non-treated cells, and total OD means OD of supernatant from completely lysed cells treated by one drop of 100% Triton X-100. LDH assay was performed in triplicate for each cell line, and each triplicate experiment was done twice, and then the mean \pm standard error was calculated.

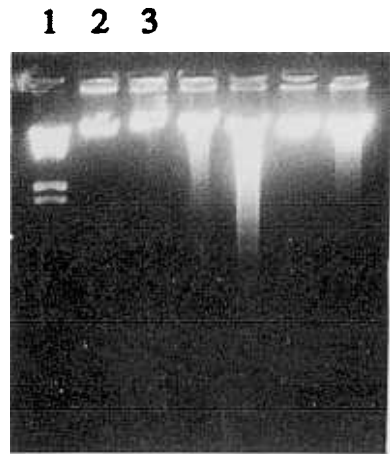


Fig. 1. DNA agarose gel electrophoresis of SK-Hep-1 cells and PLC/PRF/5 cells. Cells were treated with or without 0.01% Triton X-100 for various time intervals. λ DNA/Hind III marker (lane 1). Untreated control SK-Hep-1 cells (lane 2). SK-Hep-1 cells treated with Triton X-100 for 30 min (lane 3), 90 min (lane 4), 150 min (lane 5). Untreated control PLC/PRF/5 cells (lane 6). PLC/PRF/5 cell treated with Triton X-100 for 60 min (lane 7).

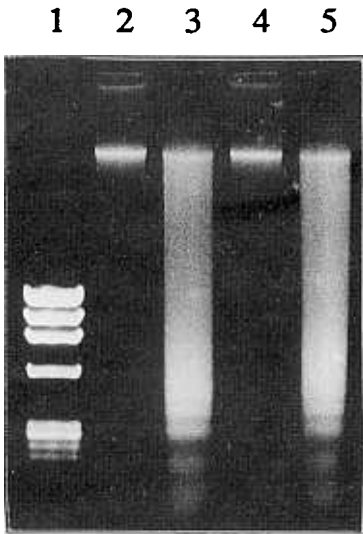


Fig. 2. DNA agarose gel electrophoresis of Hep 3B cells and Hep G2 cells. Cells were treated with or without 0.01% Triton X-100 for 150 min. ϕ X-174 RF DNA/Hae III marker (lane 1). Untreated control Hep 3B cells (lane 2). Hep 3B cells treated with Triton X-100 (lane 3). Untreated control Hep G2 cells (lane 4). Hep G2 cells treated with Triton X-100 (lane 5).

Triton X-100 induced internucleosomal DNA fragmentation typical for apoptosis. These results strongly suggest that Triton X-100 induces apoptosis in human hepatoma cell lines.

DNA fragmentation was also confirmed by TUNEL test. The TUNEL test is based on in situ labelling immunohistochemistry which detects the 3'-OH DNA ends generated by DNA fragmentation and labelled with digoxigenin-dUTP (Gavrieli *et al.* 1992). Microscopically scattered tumor cells showed strong, brown, positive staining in their nuclei and some of them showed fragmented nuclei (Fig. 3).

Morphological features of cell death induced by Triton X-100

To confirm apoptosis in morphological criteria, H & E stain was performed. Microscopically, the SK-Hep-1 cells were large epithelial tumor cells with abundant cytoplasm and showed remarkable pleomorphism. The area of zonal necrosis was not evident. However, scattered individual cells showing

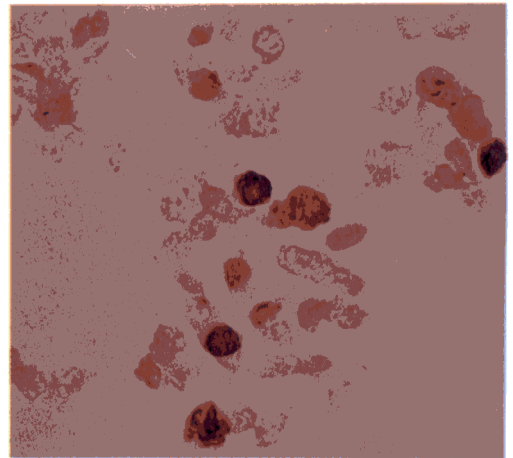


Fig. 3. TUNEL test for SK-Hep-1 cells. Many tumor cells show positive reaction in the nuclei.

fragmented nuclear and condensed cytoplasmic contents were frequently evident (Fig. 4).

Apoptosis in hepatocellular carcinoma tissues

We also evaluated the TUNEL test using resected hepatocellular carcinoma tissues embedded in paraffin blocks. We evaluated the area of no necrosis to find tumor cells in apoptosis as evidenced by positive staining in their nuclei. The frequency of positive reaction was remarkably variable between tumors. In some tumors, many of the tumor cells showed apoptosis while in others, apoptosis was rarely observed (Fig. 5).

Measurement of cell death induced by Triton X-100

To quantitate the apoptotic cell death induced by Triton X-100, the percentage of cytotoxicity was measured by LDH assay. In this experiment, LDH activity was measured by enzymatic test through the conversion of lactate to pyruvate. Fig. 6 shows that Triton X-100 concentration of 0.01% was enough to kill more than 90% of the hepatoma cells tested.

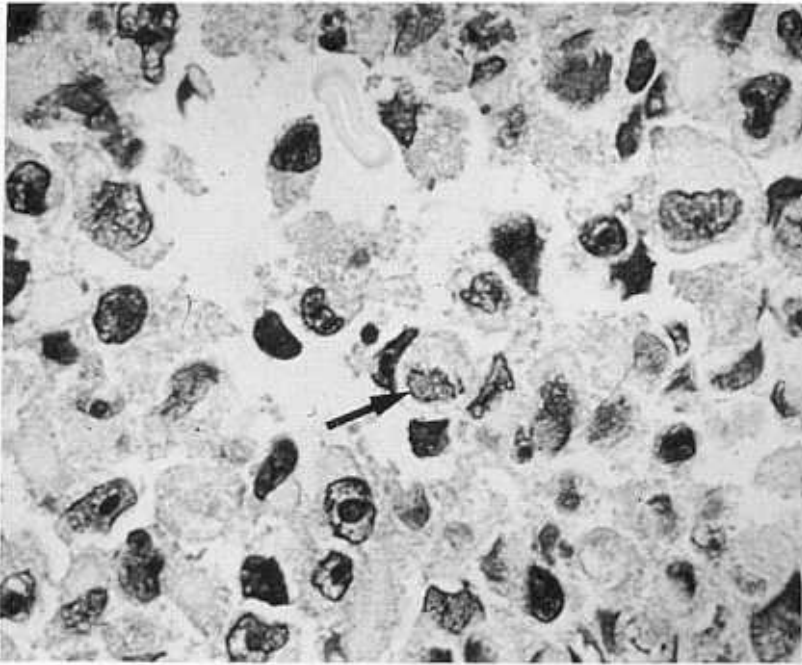


Fig. 4. Microscopic features of SK-Hep-1 cells. Most of tumor cells have large nuclei and abundant cytoplasm. Some of the tumor cells show fragmented nuclei (arrow).

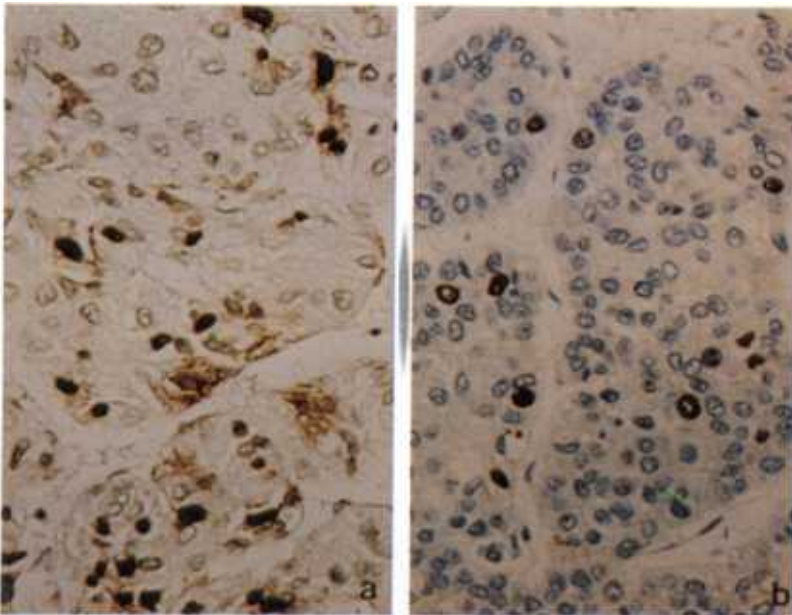


Fig. 5. TUNEL test for the hepatoma tissues. Some of the tumor cells show positive reaction in the nuclei. Considerable differences in the amount of positive reaction are evident between tumors. (a) Some tumors frequently show TUNEL positive tumor cells and (b) some tumors occasionally show TUNEL positive tumor cells.

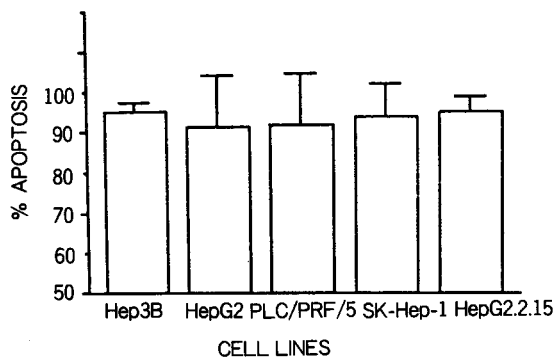


Fig. 6. Cell death induced by Triton X-100 in hepatoma cell lines. After overnight incubation of 10^5 cells/well, cells were treated with 0.01% Triton X-100 for 15 min. Optical density of the reaction mixtures of LDH substrate mixture added to the culture supernatant was measured at 490 nm. Percent cytotoxicity was calculated. Each value indicates mean \pm S.E. of 6 independent experiments.

DISCUSSION

Cell death may occur by either of two mechanisms: necrosis or apoptosis. Apoptotic cell death in normal tissue is a biological phenomenon that maintains homeostasis of the body systems under physiological conditions. Apoptotic cell death plays a major role in the regulation of cell growth and this process may be activated by various agents through different pathways (Clarke, 1990; Ellis, 1992; Raff, 1992; Vaux, 1993; Wyllie, 1993). Apoptosis due to a specific inducing stimulus is considered to be an actively regulated cell response. A variety of highly selective cytotoxic agents induce apoptosis only in those cells having relevant response pathways. Numerous morphological features and biochemical parameters were reported in the induction of apoptosis. Apoptosis is defined by the occurrence of characteristic morphological changes such as chromatin and cytoplasmic condensation, which are typical morphological results due to internucleosomal DNA fragmentation. These features distinguish apoptosis from passive necrotic death (Wyllie, 1992; Vaux, 1993). However, despite intensive research, the mechanisms by which the induction of apoptosis

causes cell death are still poorly defined. Although the dysregulation of physiological signals and related mechanisms controlling cell proliferation have been a major focus in cancer research, recent evidence suggests that explicit evaluation of apoptosis may be equally important in understanding multistage carcinogenesis (James and Muskhelishvili, 1994).

We report here that Triton X-100 induces cell death in various hepatoma cell lines. We assessed the type of cell death in human hepatoma cell lines on the basis of morphological and biochemical characteristics after treatment with 0.01% Triton X-100. DNA agarose gel electrophoresis showed a typical DNA fragmentation ladder pattern. DNA extracted from Triton X-100 treated hepatoma cells showed a ladder pattern consisting of multimers of 180 to 200 bp, indicating extensive DNA cleavage into oligonucleosomal units by an endogenous endonuclease. This result strongly suggests that Triton X-100 induces apoptosis in hepatoma cells. Dying cells, which detached from the cell monolayer, showed morphological characteristics of apoptosis such as chromatin condensation, nuclear disintegration and cellular fragmentation into eosinophilic globules. These results provide strong evidence that the cell death induced by Triton X-100 in human hepatoma cells is mainly apoptotic. The existence of many apoptotic cells, which are morphologically characterized by chromatin margination, was also seen in the hepatocellular carcinoma tissues. From these findings we conclude that apoptosis is frequent in the sporadic hepatocellular carcinomas in some patients, however the degree of apoptosis between those tumors is remarkably different.

It has been reported that Triton X-100 induced cell death showed similarities to the pattern of cell death induced by cytotoxic lymphocytes. In both systems, apoptotic cell death is dependent on extracellular calcium (Hameed *et al.* 1989; Shi *et al.* 1992; Borner *et al.* 1994). The cytotoxic lymphocyte granule proteins such as perforin, and serine proteases such as fragmentin and granzyme, are responsible for the induction of apoptotic cell death. The serine proteases mediate apoptotic morphology and DNA fragmentation while perforin facilitates serine proteases uptake or directly perforating the target cell membrane (Zychlinsky *et al.* 1991; Shi *et al.* 1992; Shiver *et al.* 1992). However, it was

also reported that perforin alone can induce apoptosis (Hameed *et al.* 1989). Since Triton X-100 is widely used to permeabilize cell membrane, it might be possible that Triton X-100 displays activity similar to perforin by pore formation in the cell membrane. Apoptosis induced by Triton X-100 appeared within less than 60 min. Similar observations were recently described for the induction of apoptosis in thymoma cells given cold shock (Kruman *et al.* 1992), and cell lines of prostate cancer and colorectal cancer treated with Triton X-100 (Borner *et al.* 1994). The extremely fast induction of apoptosis in our experiment makes the following explanation possible: 1) the cellular components required for apoptosis already in place were activated by Triton X-100. 2) Inhibitors of an active apoptotic pathway were denatured by Triton X-100. It is also possible that the depletion of serum primed the cells for apoptosis. A further study would be necessary to verify the above considerations.

Here we show that the non-ionic detergent Triton X-100 caused the distinct morphology and internucleosomal DNA fragmentation typical for apoptosis in human hepatoma cell lines. We demonstrate extensive chromatin cleavage into oligonucleosome-length fragments (DNA ladder) in various human hepatoma cell lines, which suggests the activation of an endogenous endonuclease previously found to be involved in the process of apoptosis. We also showed that more than 90% of hepatoma cells were dead by apoptosis induced by 0.01% Triton X-100. The clarification of the mechanism of Triton X-100 induced apoptosis will shed further light of the essential components involving the apoptotic program and their regulation in hepatoma cell lines.

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